Zoosyst. Evol. 101 (2) 2025, 791–804 | DOI 10.3897/zse.101.137781

Taxonomic status and phylogenetic analyses based on complete mitochondrial genome and microscopic ossicles: Redescription of a controversial tropical sea cucumber species (Holothuroidea, *Holothuria* Linnaeus, 1767)

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Academic editor: Pavel Stoev ♦ **Received** 24 September 2024 ♦ **Accepted** 19 March 2025 ♦ **Published** 15 April 2025

Abstract

To explore the taxonomic status of a tropical sea cucumber species with controversy, we conducted an investigation into its morphological characteristics (external form and ossicles) and mitochondrial genome and rearranged the historical materials of the species and its confusing species. The species was first described in the middle of the 18th century but later synonymized with *Bohadschia ocellata* Jaeger, 1833, *Holothuria hamata* Pearson, 1913, or *Holothuria kurti* Ludwig, 1891, by different scholars. The observed ossicles encompassed multiple types, including buttons, C-shaped ossicles, rods, and tables, with a distinct subtype identified as tack-like tables. Ventral and dorsal body walls exhibited similar table and button ossicle shapes. The mitogenome contained 13 protein-coding genes (PCGs), 2 ribosomal RNAs (rRNAs), and 22 transfer RNA (tRNA) genes, with a sequence length of 15,797 bp. Subsequent construction of a phylogenetic tree using Bayesian inference (BI) and maximum likelihood (ML) methods indicated that the species was grouped into a separate branch firstly with *Holothuria* (*Theelothuria*) *spinifera* Théel, 1886, so suggesting its taxonomic classification under subgenus *Theelothuria* Deichmann, 1958. Based on the data of this study and previous literature, we suggested that the Latin name for this species should tentatively be "*Holothuria ocellata* Jaeger, 1833 *sensu* Théel 1886." The morphologic and genomic information in the current study could be valuable in further biological studies of this sea cucumber species.

Key Words

Mitochondrial genome, molecular phylogenetics, ossicles, taxonomy, tropical sea cucumber

Introduction

Holothuroidea, commonly known as holothuroids or sea cucumbers, includes about 1700 species worldwide (Reich 2017; Harini et al. 2024). These species are one of the largest and most diverse groups of benthic invertebrates in the intertidal and subtidal zones, constituting the vast majority of total biomass in slope and abyssal benthic systems (Slater and Chen 2015). Within this class, the family Holothuriidae comprises 45 genera and approximately 778 species (WoRMS 2023a), and it has also emerged as one of the most significant benthic taxa due to its substantial economic and ecological value in aquaculture, pharmacy, or high biodiversity (Purcell et al. 2012; Soliman et al. 2019). However, due to morphological similarities or the brevity of descriptions in some previous literature predating the mainstream adoption of Linnaean classification, the taxonomic status of certain species has long been subject to dispute.

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Sea cucumber *Holothuria ocellata* Jaeger, 1833, also known as *Bohadschia ocellata* Jaeger, 1833, is a subject of controversy. This species inhabits the Indo-West Pacific region and exhibits a wide bathymetric distribution from 0 to 270 m (Clark and Rowe 1971; Guille et al. 1986; Liao 1997; Teo and Ng 2009). While some authors asserted it belongs to the genus *Holothuria* Linnaeus, 1767 (Heding 1939; Liao 1980; Liao 1997), others recognized it as a member of the genus *Bohadschia* Jaeger, 1833 (Kim et al. 2013; Patantis et al. 2019). Still others argued that *H. ocellata* is synonymous with other sea cucumber species like *Holothuria hamata* Pearson, 1913, or *Holothuria kurti* Ludwig, 1891 (Aydin et al. 2019; Moazzam and Moazzam 2020; Purcell et al. 2023). The databases that are widely referenced present different results. WoRMS (https://www.marinespecies.org/) acknowledges the validity of *B. ocellata*, but the NCBI taxonomy database (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser) recognizes the validity of *H. ocellata*. Before reaching a conclusion, we use the appellation of "*Holothuria ocellata*" in this study.

Besides the external morphology, the traditional morphological classification of sea cucumbers is mainly based on the structure of ossicles within their body wall, which is part of the sea cucumber's calcified endoskeleton (Aydin and Erkan 2015). However, due to the variability and multiformity of ossicles within the same species, the morphological analysis often leads to the wrong species identification of holothuroids (Massin et al. 2000; Utzeri et al. 2020). In addition, hybridization events among related species may also lead to animals with mixed morphological features, which might add confounding factors (Uthicke et al. 2005; Yoshida et al. 2012; Kim et al. 2013). Thus, to improve the accuracy of species identification, molecular information is necessary to complement and ascertain the taxonomic status of sea cucumbers (Dettaï et al. 2011; Aydin and Erkan 2015; Valente et al. 2015). Among molecular technologies, the mitochondrial genome is an excellent tool for species identification, which has been widely utilized in the work of sea cucumber species identification (Utzeri et al. 2020; Sun et al. 2021; Ma et al. 2022; Li et al. 2022).

In this study, as a first step for identifying the species of obtained samples, we provided a detailed description of the external morphology and ossicle structure for *H. ocellata* using microscopy inspection. Then, we sequenced the complete mitochondrial genome of this species and compared it with the other complete mitochondrial sequences of other holothuroid species. Through integrating morphological observation and molecular biological methods, along with comparison to other literature, we aim to clarify the taxonomic history and position of this sea cucumber species. The produced molecular and morphological results have addressed a gap in the phylogenetic analysis of *H. ocellata* and have provided important classification and identification data for this species.

Materials and methods

Sample collection and preservation

The sea cucumber sample (Fig. 1) was collected by diving from the coastal waters of Wenchang in China in August 2022 and was transported alive to the laboratory in fresh seawater. The sample was identified based on morphological characteristics and DNA barcoding before alcohol preservation, and the individual was chosen for mitogenome sequencing. Muscle tissues were placed in 95% ethanol at -20 °C for further study.

Microscopic analyses of the ossicles and calcareous ring

To verify the identity of the specimen, both optical microscopy and scanning electron microscopy were used to observe and analyze the types and sizes of ossicles in the body wall, tentacle, tube feet, and papillae. The ossicles were extracted using the method modified by Teo and Ng (2009). Three tissue pieces weighing about 1 g were collected from each target site and rinsed with distilled water. Then, 8% sodium hypochlorite was added to each sample and shocked to digest for approximately 3–5 min. After white particles appeared at the bottom of the tube, undigested tissue pieces were picked out and left to stand for 5 minutes. An appropriate amount of turbid liquid was absorbed with white particles and placed on a slide. We used the light microscope to preliminarily observe these samples and confirmed different types of ossicles. Then, the other turbid liquid was dropped on the conductive tape and stoved in a drying oven, followed by gold spray treatment with an ion sputtering apparatus. A scanning electron microscope (SEM; HITACHI, S-4800) was used to observe and photograph these samples. The size of the ossicles in the photographs was measured by the soft ruler and converted to actual lengths based on the scale bars. Ten individuals from each ossicle were randomly selected for measurement. The calcareous ring was observed and depicted with the stereoscopic microscope. The size of the calcareous ring in the photographs was measured by the soft ruler and converted to actual lengths based on the scale bars.

DNA extraction and sequencing

According to the manufacturer's protocol, the total DNA of the sea cucumber was extracted using the TIANamp Marine Animal DNA Kit (TIANGEN, Beijing, China). The DNA library was sequenced by Origingene Co., Ltd. (Shanghai, China) using the Illumina NovaSeq™ 6000 platform with an insert size of 300–500 bp. Approximately 9.09 GB of raw data from *Holothuria ocellata* were generated with 150-base-pair paired-end read lengths. The quality control and assessment of data

Figure 1. Dorsal (**A**) and ventral (**B**) views of *H. ocellata*.

were used by Cutadapt v1.16 and FASTQC v0.11.4, respectively (Martin 2011). The mitogenome was assembled by NOVOPLASTY v4.2 with default parameters (Dierckxsens et al. 2017).

Sequence annotation and analysis

The mitogenome of *H. ocellata* was annotated by the MITOS WEBSERVER (Donath et al. 2019) (http://mitos2.bioinf.uni-leipzig.de/index.py). The graphical circle map of the mitogenome was drawn using the online PROKSEE tool (Stothard et al. 2019) (https://proksee.ca/). The relative synonymous codon usage (RSCU) was conducted using CODONW v1.4.4 (http://codonw.sourceforge.net). The blank region of the mitogenome was defined as the control region (D-loop) and compared with the mitogenome of the reference species to determine the control region. The TRNASCAN-SE tool was used to identify the tRNA gene and predict the secondary structure diagram of tRNA (Chan and Lowe 2019). The composition skewness of each segment was calculated by the following formulas: AT-skew = (A − T) / (A + T); GC-skew = (G − C) / (G + C) (Perna and Kocher 1995).

Phylogenetic analysis

Besides the sequence obtained in this study, mitogenomes of 35 previously sequenced sea cucumber species in the class Holothuroidea and 2 previously sequenced starfish species in the class Asteroidea (with the latter as the outgroup taxon) were used in the phylogenetic analyses. We extracted the nucleotide sequences of the 13 protein-coding genes from each mitogenome as the dataset to construct the phylogenetic tree. Sequences were extracted and concatenated by PHYLOSUITE v1.2.3 (Zhang et al. 2020; Xiang et al. 2023), and these sequences were aligned and refined through MAFFT v7.505 and MACSE v2.06 (Katoh and Standley 2013; Ranwez et al. 2018). Ambiguously aligned fragments of 13 alignments were removed in batches based on GBLOCKS with the default parameter settings (Talavera and Castresana 2007). MODELFINDER v2.2.0 was used to select the best-fit model through the BIC criterion (Kalyaanamoorthy et al. 2017). Maximum likelihood (ML) phylogenies were inferred by IQ-TREE v2.2.0 under the model automatically selected by the 'Auto' option for 20000 ultrafast bootstraps (Guindon et al. 2010; Minh et al. 2013; Nguyen et al. 2015). Bayesian inference (BI) phylogenies were inferred through MRBAYES v3.2.7a under the GTR+I+G+F model (2 parallel runs, 200000 generations), with the initial 25% of sampled data discarded as burn-in (Ronquist et al. 2012).

To determine whether the species belongs to the genus *Bohadschia* or to *Holothuria*, we selected the mitochondrial sequence fragments of all *Bohadschia* and some *Holothuria* from NCBI to perform the phylogenetic tree. 13 *rrnL* genes and 9 *cox1* genes, including sequences from this study, were selected to build Neighbor-Joining (NJ) phylogenetic trees based on two genes, respectively. Sequences were aligned by SEQMAN from DNASTAR software (USA). The trees were constructed, and the distances between and within groups were determined by MEGA v5.0 (Tamura et al. 2011) with 1000 bootstrap replicates based on genetic distances calculated with the Kimura-2-parameter (K2P) model by the same software.

Results

Morphological characters

The specimen, in the state of natural extension, is 24.35 ± 0.3 cm long and 5 ± 0.2 cm wide. The body is cylindrical, tapering at both ends. The bivium presents convex (Fig. 1A), while the trivium presents flattened (Fig. 1B). The mouth is ventral, and the anus is located in a terminal. The dorsal color is grayish on both sides and yellowish-brown in the middle, interspersed with tan spots. The whole dorsal surface is covered with conic papillae, which are milky at the tip and brown at the base. Each papilla is usually surrounded by a narrow light-grey band and a white band from inside to outside, although the white band may be absent or non-significant in some papillae. The ventral has a lighter color and slightly flatter shape than the dorsal. The ventral papillae are arranged in 3–4 bands, concentrated in the middle of the trivium, showing a trend of spreading to the interambulacrum on both sides. Papillae on the sides of the ventral are prominent in size, arranged along the sides of the body to form the dorsoventral junction with a number of 41 or 42 on each side.

Microscopic analyses of the ossicles and calcareous ring

The body wall of the specimen has four major ossicle types, including multiple buttons, C-shaped ossicles, rods, and tables. Ventral/dorsal body walls have similar table and button types. The majority of the buttons are knobbed, yet the minority are smooth and nearly oval, usually with 3 pairs of holes, 30–68 μm in length and 30–40 μm in width (Fig. 2A, B). Some buttons are long ellipsoids with up to 4–7 pairs of holes, 63–88 μm in length, and 27–38 μm in width (Fig. 2C, D). The paired holes of most buttons are symmetrical and similar in size to each other. Tables are intact with nearly circular discs with smooth or wavy margins. There are basically two types of typical tables: 1) the disc is convex with a larger size of 81–136 μm in diameter, with dozens of holes in the circumferential margin; the spire is moderate in length, relatively thin, and terminating in a cluster of spines (Fig. 2E, F); 2) the disc is convex with a smaller size, 64–82 μm in diameter, with 8–12 holes in the circumferential margin; the spire is moderate in length, relatively thick, and terminating in a cluster of spines (Fig. 2G, H). The rods in the body wall are commonly composed of simple spicules, which are smooth and elongated, with a wide range of length variations, 131–496 μm long (Fig. 2I). The C-shaped ossicles in the body wall are commonly C-form, smooth, and elongated, with a wide range of length variations, 200–300 μm long (Fig. 2K). Tentacles with spiny rods, 232–376 μm long (Fig. 2P–R).

Ossicles of papillae and tube feet include rods, perforated plates, and tables. The morphology of rods is diverse and complex, with spines present or not and thicknesses different (Fig. 2I–J). Most perforated plates, measuring up to 200 μm in length, have perforations in the middle, and some plates are perforated throughout their whole length (Fig. 2L–O). The type of tables in papillae is basically consistent with those of the body wall or tube feet, except for the tack-like tables. The tack-like tables, only found in papillae, with concave bottoms, are few in number, standing up to 240 μm high, and have a tall attenuating spire with several cross-beams, resembling the shape of a nail (Fig. 2S, T).

Calcareous ring, with radial plates obviously larger than interradial ones; radial plates with anterior concave and obviously posterior bifurcation; interradial plates with cuspate anteriorly (Suppl. material 1).

Mitogenome composition and characteristics

The complete mitochondrial genome of the sea cucumber (GenBank No. OQ731944) is 15,797 bp in length (Fig. 3). The mitogenome contains 37 typical genes (13 PCGs, 22 tRNAs, and 2 rRNAs) and 2 control regions. Most mitochondrial genes are encoded on the H-strand, except for nad6 and 5 tRNA genes (*trn*S2, *trn*Q, trnA, *trn*V, and *trn*D) that are encoded on the L-strand (Table 1). The nucleotide composition of mitogenomes has a higher A+T bias of 59.01%, and all PCGs show negative GC-skew except for *nad6* (Table 2).

The number of PCGs in mitogenomes is consistent with the general findings on the mitogenomes in Holothuriidae species. Twelve PCGs (*cox1*, *cox2*, *cox3*, *atp8*, *atp6*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4l*, *nad5*, and *cob*) are coded on the heavy strand (H-strand), while the remaining one (*nad5*) is coded on the light strand (L-strand). All 13 PCGs collectively encode 3696 amino acids. All PCGs use the initiation codon ATG. The termination codons TAA and TAG are commonly observed, although the incomplete termination codon T is found in *cox2* and *nad4* in the mitogenome.

We calculated the relative synonymous codon usage (RSCU) of the mitogenome (Table 3, Fig. 4), and the results showed that the frequency of NNA and NNC (N represents A, T, C, G) is higher than NNT and NNG. The most frequent amino acids in the coding sequences of mitochondrial proteins are Leu1, Phe, and Ile (> 290) (Table 3). Moreover, the minimally used amino acid in the mitogenomes is Trp (< 30).

Similar to the most holothuroids, the mitogenome of *Holothuria ocellata* has one *rrnS* (12S rRNA) and one *rrnL* (16S rRNA) gene. The *rrnS* gene is located between *trnF* and *trnE*, and the *rrnL* gene is located between *nad2* and *cox1*. In the *H. ocellata* mitogenome, the A+T content of rRNA is 59.82%. The AT-skew of rRNA is strongly positive, whereas the GC-skew is slightly negative, indicating that the contents of A and C are higher than those of T and G in the rRNA, respectively. And there are

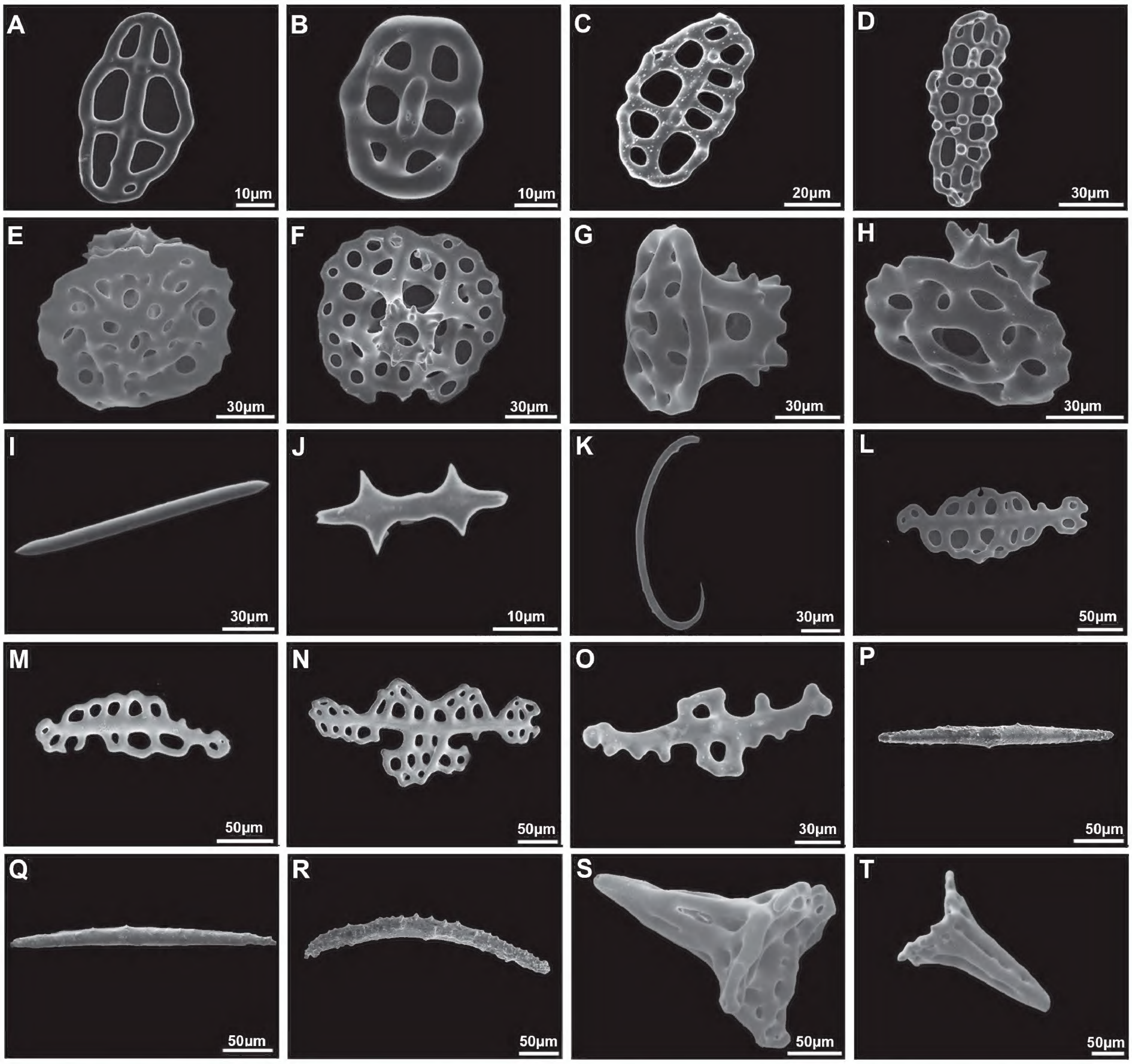

Figure 2. *H. ocellata*: scanning electron microscopy images of ossicles. **A, B.** Buttons with 3 pairs of holes; **C, D.** Buttons with 4–7 pairs of holes; **E, F.** Tables with larger discs; **G, H.** Tables with smaller discs; **I, J.** Rods in papillae, tube feet, or body wall; **K.** C-shaped ossicles in the body wall; **L-O.** Perforated plates of papillae, or tube feet; **P–R.** Rods in tentacles; **S, T.** Tack-like tables of papillae.

22 tRNA genes. The secondary clover-leaf structures of tRNA genes identified in the mitogenome are shown in Fig. 5. These tRNA genes vary in length from 57 bp to 75 bp. All predicted tRNAs display the typical clover-leaf secondary structure, except for *trnS1*.

Phylogenetic analysis

Phylogenetic relationships are constructed based on the sequences of 13 PCGs of 38 mitogenomes using BI and ML methods. The phylogenetic trees constructed by the two methods are consistent with high intermediate bootstrap values, and the topological structure of the trees is entirely the same (Fig. 6A). The results showed that 10 *Holothuria* sequences formed a monophyletic group in both ML and BI analyses. Moreover, *H. ocellata* formed a sister group with *Holothuria* (*Theelothuria*) *spinifera* Théel, 1886, and both divided with *Bohadschia argus* Jaeger, 1833.

Using the NJ method, the unrooted phylogenetic trees of the *rrnL* and *cox1* genes both indicated that *Holothuria* and *Bohadschia* species form a monophyletic group, respectively (Fig. 6B, C). *Holothuria* and *Bohadschia* sequences are observably divided from each other. Besides, the tree of *rrnL* showed the *Holothuria ocellata* sequence obtained in this study clusters with the *H. ocellata* sequence from NCBI. And the tree of *cox1* showed the *H. ocellata* sequence is divided with *Bohadschia ocellata* sequences from NCBI.

Table 1. Summary of the genes in the *H. ocellata* mitogenome.

Gene	Strand	Location	Size (bp)	Start codon	Stop codon	Anticodon
cox1	H	1-1557	1557	ATG	TAG	
trnR	H	1564-1632	69			TCG
nad4l	H	1633-1929	297	ATG	TAA	
cox2	H	1931-2618	688	ATG	T	
trnK	H	2619-2686	68			CTT
atp8	H	2687-2854	168	ATG	TAA	
atp6	H	2884-3531	648	ATG	TAA	
cox3	H	3534-4316	783	ATG	TAA	
trnS2	L	4315-4385	71			TGA
nad3	H	4404-4748	345	ATG	TAA	
nad4	H	4753-6109	1357	ATG	T	
trnH	H	6111-6176	66			GTG
trnS1	H	6189-6245	57			GCT
nad5	H	6477-8081	1605	ATG	TAA	
nad6	L	8100-8588	489	ATG	TAG	
cob	H	8597-9739	1143	ATG	TAG	
trnF	H	9742-9812	71			GAA
rrnS	H	9812-10633	822			
trnE	H	10631-10699	69			TTC
trnT	H	10701-10769	69			TGT
trnP	H	11252-11319	68			TGG
trnQ	L	11316-11385	70			TTG
trnN	H	11386-11455	70			GTT
trnL1	H	11456-11527	72			TAG
trnA	L	11527-11595	69			TGC
trnW	H	11596-11665	70			TCA
trnC	H	11666-11727	62			GCA
trnV	L	11728-11797	70			TAC
trnM	H	11816-11885	70			CAT
trnD	L	11891-11960	70			GTC
trnY	H	11961-12028	68			GTA
trnG	H	12028-12102	75			TCC
trnL2	H	12103-12173	71			TAA
nad1	H	12174-13145	972	ATG	TAG	
trnI	H	13168-13235	68			GAT
nad2	H	13236-14279	1044	ATG	TAA	
rrnL	H	14243-15665	1423			

Table 2. Compositions and skewness of *H. ocellata* mitogenomes.

Region	A%	T%	G%	C%	A + T (%)	G + C (%)	AT-Skew	GC-Skew
whole	31.47	27.54	16.46	24.52	59.01	40.98	0.067	-0.197
PCGs	28.73	29.70	16.54	25.03	58.43	41.57	-0.017	-0.204
cox1	27.87	27.49	18.24	26.40	55.36	44.64	0.007	-0.183
nad4l	28.62	30.64	11.45	29.29	59.26	40.74	-0.034	-0.438
cox2	31.24	24.96	16.59	27.20	56.20	43.79	0.112	-0.242
atp8	37.50	27.98	13.69	20.83	65.48	34.52	0.145	-0.207
atp6	31.33	28.09	12.96	27.62	59.42	40.58	0.055	-0.361
cox3	27.33	27.59	17.75	27.33	54.92	45.08	-0.005	-0.212
nad3	29.28	27.25	16.52	26.96	56.53	43.48	0.036	-0.240
nad4	31.73	25.88	15.06	27.34	57.61	42.40	0.102	-0.290
nad5	29.78	30.78	16.01	23.43	60.56	39.44	-0.016	-0.188
nad6	17.18	45.40	25.56	11.86	62.58	37.42	-0.451	0.366
cob	28.26	30.62	16.36	24.76	58.88	41.12	-0.040	-0.204
nad1	26.34	32.82	16.87	23.97	59.16	40.84	-0.110	-0.174
nad2	28.93	31.51	15.61	23.95	60.44	39.56	-0.043	-0.211
tRNAs	31.06	28.42	21.02	19.50	59.48	40.52	0.044	0.038
rRNAs	37.59	22.23	18.98	21.20	59.82	40.18	0.257	-0.055

Table 3. Codon numbers and relative synonymous codon usages in 13 PCGs of *H. ocellata*.

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	143	0.97	UCU(S)	53	1.18	UAU(Y)	44	0.85	UGU(C)	18	1.00
UUC(F)	151	1.03	UCC(S)	86	1.92	UAC(Y)	60	1.15	UGC(C)	18	1.00
UUA(L)	138	1.34	UCA(S)	71	1.58	UAA(*)	7	0.22	UGA(*)	82	2.59
UUG(L)	28	0.27	UCG(S)	18	0.40	UAG(*)	6	0.19	UGG(W)	23	1.00
CUU(L)	108	1.05	CCU(P)	46	1.07	CAU(H)	17	0.42	CGU(R)	9	0.37
CUC(L)	92	0.89	CCC(P)	46	1.07	CAC(H)	64	1.58	CGC(R)	6	0.24
CUA(L)	209	2.03	CCA(P)	70	1.63	CAA(Q)	56	1.38	CGA(R)	49	2.00
CUG(L)	42	0.41	CCG(P)	10	0.23	CAG(Q)	25	0.62	CGG(R)	9	0.37
AUU(I)	78	0.64	ACU(T)	53	0.87	AAU(N)	31	0.81	AGU(S)	15	0.33
AUC(I)	61	0.50	ACC(T)	89	1.45	AAC(N)	46	1.19	AGC(S)	26	0.58
AUA(I)	226	1.86	ACA(T)	88	1.44	AAA(K)	98	1.26	AGA(R)	68	2.78
AUG(M)	74	1.00	ACG(T)	15	0.24	AAG(K)	57	0.74	AGG(R)	6	0.24
GUU(V)	63	1.18	GCU(A)	62	0.94	GAU(D)	17	0.55	GGU(G)	26	0.47
GUC(V)	57	1.07	GCC(A)	121	1.83	GAC(D)	45	1.45	GGC(G)	39	0.70
GUA(V)	79	1.48	GCA(A)	70	1.06	GAA(E)	63	1.29	GGA(G)	110	1.98
GUG(V)	15	0.28	GCG(A)	12	0.18	GAG(E)	35	0.71	GGG(G)	47	0.85

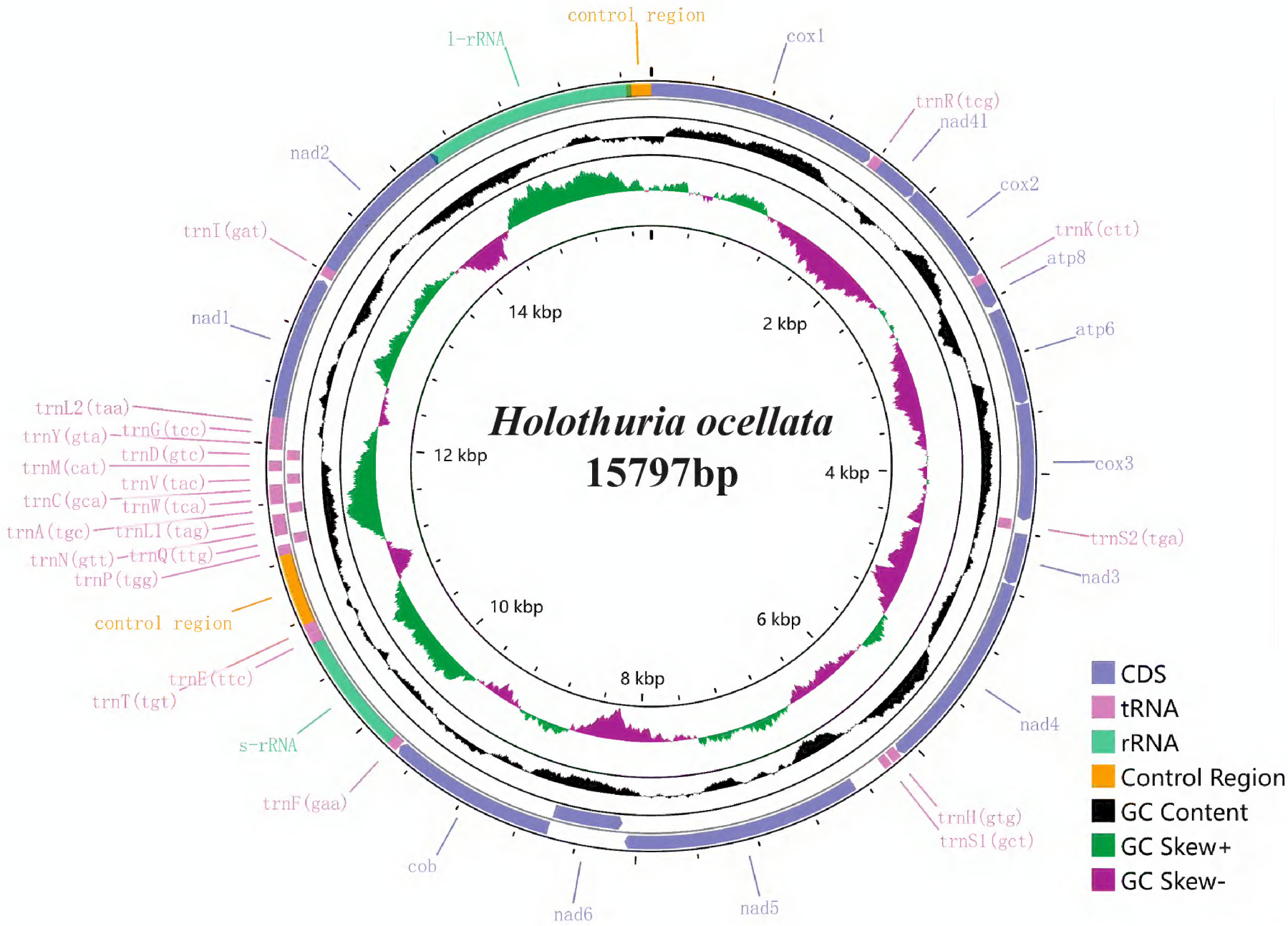

Figure 3. Mitochondrial genome maps of *H. ocellata*. Genes encoded on the heavy or light strands are shown outside or inside the circular gene map, respectively.

Discussion

Comparative analysis of suspected synonyms

vs. B. ocellata Jaeger, 1833

The controversy regarding the nomenclature of two sea cucumber species (*H. ocellata* / *B. ocellata*) stems from the blurry original nomenclators. Jaeger (1833) first found a sea cucumber species and classified it into the subgenus *Holothuria*, tribes *Bohadschia*, the latter being subordinate to the former, and named this species "*ocellata*." With the continuous progress of sea cucumber taxonomy, both *Holothuria* and *Bohadschia* have evolved into independent and valid genera (WoRMs 2023b, c). Due to the lack of an initial description and the continuous change

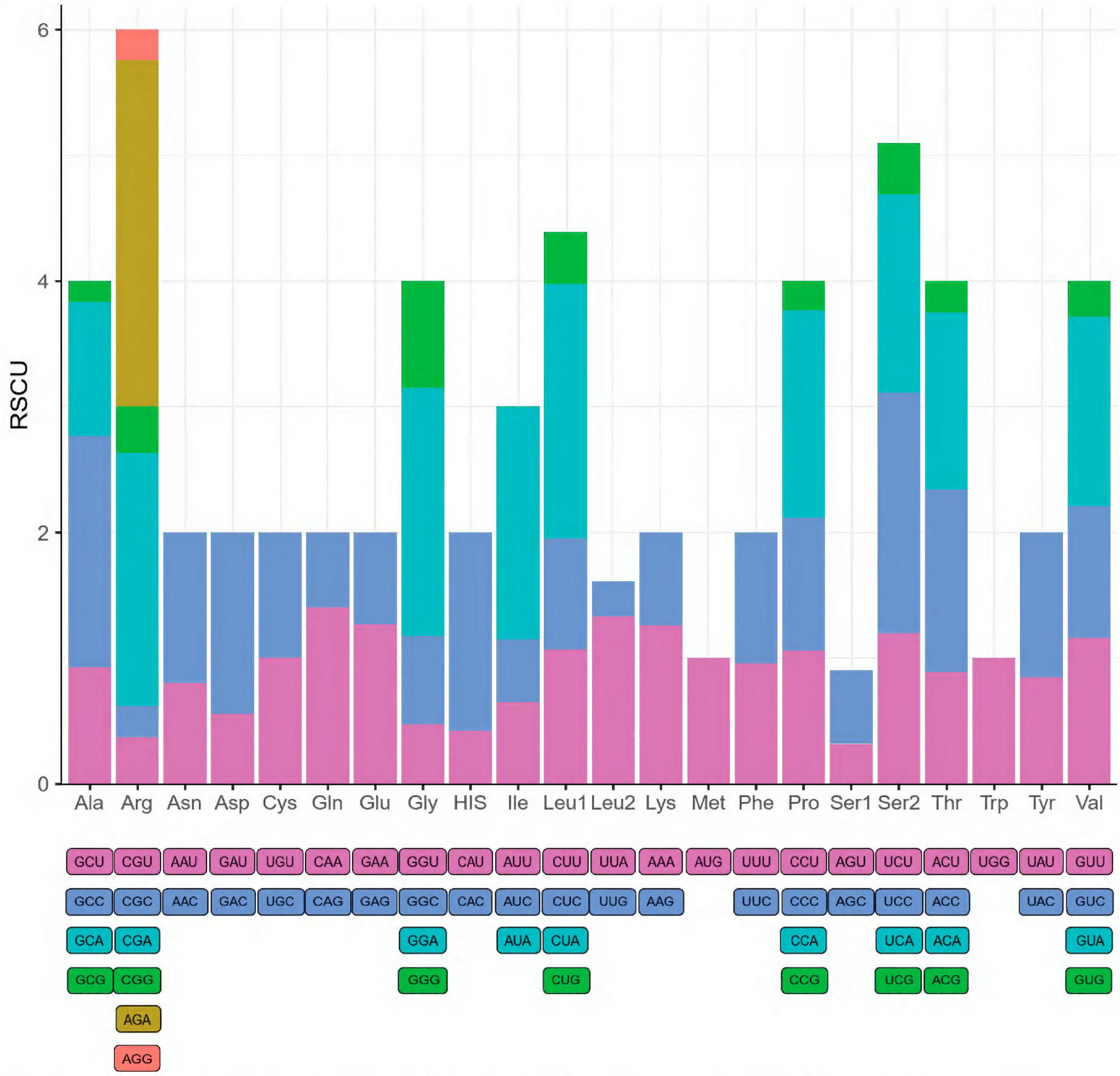
RSCU
6
4
2
0
Ala Arg Asn Asp Cys Gln Glu Gly HIS Ile Leu1 Leu2 Lys Met Phe Pro Ser1 Ser2 Thr Trp Tyr Val
GCU CGU AAU GAU UGU CAA GAA GGU CAU AUU CUU UUA AAA AUG UUU CCU AGU UCU ACU UGG UAU GUU
GCC CGC AAC GAC UGC CAG GAG GGC CAC AUC CUC UUG AAG UUC CCC AGC UCC ACC UAC GUC
GCA CGA GGA AUA CUA CCA UCA ACA GUA
GCG CGG GGG CUG CCG UCG ACG GUG
AGA
AGG

Figure 4. The relative synonymous codon usage (RSCU) in *H. ocellata* mitogenome. Codon families are labeled on the x-axis. The termination codon is not given.

of classification status, subsequent scholars had different opinions and controversies about this sea cucumber species. Some scholars continued to use *H. ocellata* as the name of the species they observed (Semper 1868; Pearson 1913; Heding 1939; Clark 1946; Rowe 1969; Liao 1980; Liao 1997; Teo and Ng 2009; Kamarudin et al. 2010). Conversely, some scholars used *B. ocellata* as the name of the species they found (Kim et al. 2013; Patantis et al. 2019; Javanmardi et al. 2020).

In this study, based on morphological characters and mitochondrial genome sequences, we believe that the two current Latin names, "*Holothuria ocellata*" and "*Bohadschia ocellata*," represent two different species, respectively. Firstly, compared with the descriptions and figures of *B. ocellata* provided by Purcell et al. (2023), these two species have obviously different ossicles and spots: 1) The types of ossicles in *H. ocellata* include buttons and tables, while *B. ocellata* has none of these; 2) Compared to *H. ocellata*, the spots of *B. ocellata* are irregular and differ greatly in size or shape. Finally, after identifying this classification problem between *H. ocellata* and *B. ocellata*, we subsequently selected the mitochondrial sequences of all *Bohadschia* and some *Holothuria* from NCBI and performed two phylogenetic trees based on *rrnL* and *cox1* genes. The result showed a clear separation between the sequences of *H. ocellata* and *B. ocellata*. Furthermore, the phylogenetic tree based on the sequences of 13 PCGs also proved that *H. ocellata* does not belong to the genus *Bohadschia*. For the above reasons, rather than synonyms, it is reasonable to consider *H. ocellata* and *B. ocellata* as two different species.

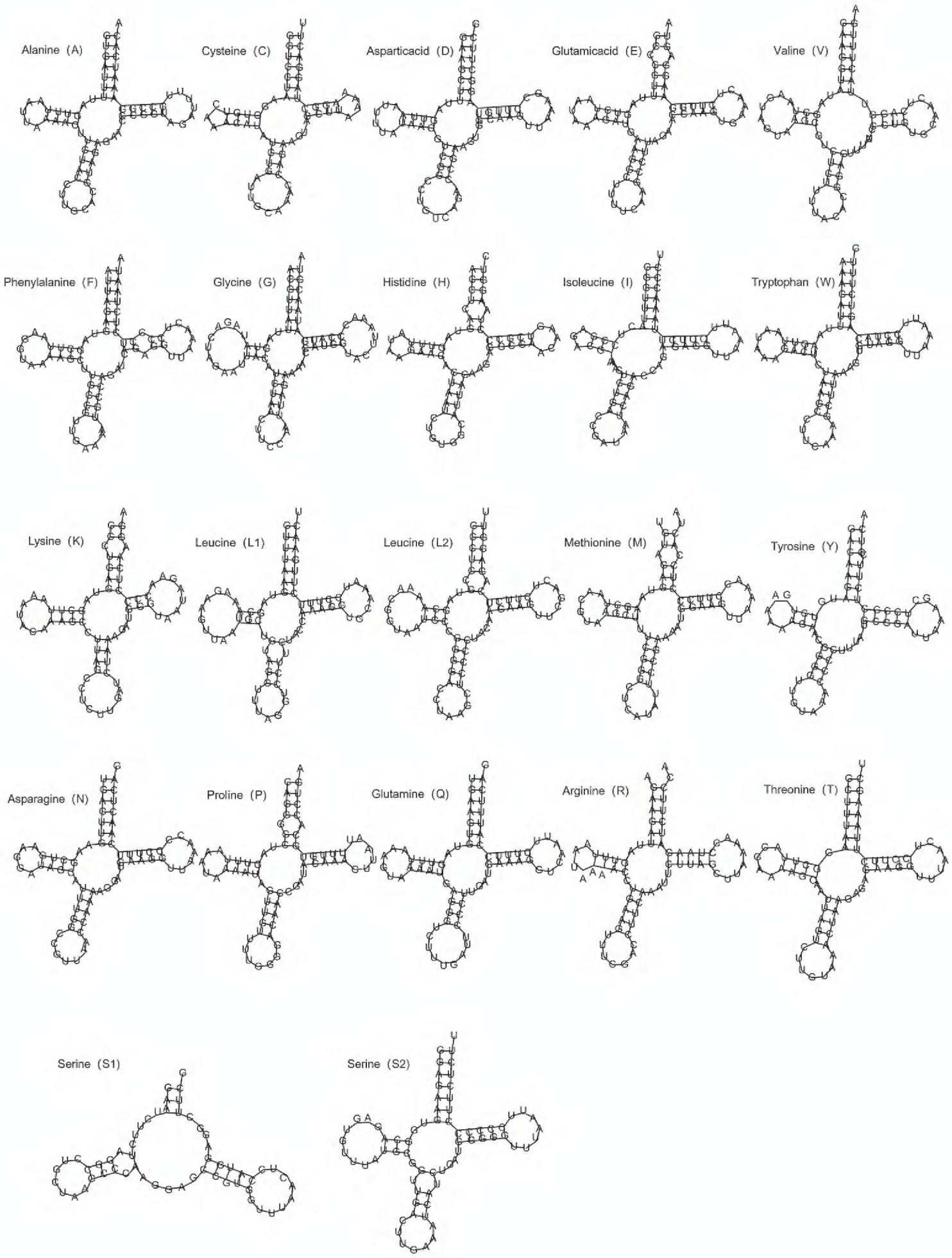

Figure 5. Inferred secondary structures of the 22 tRNA genes of *H. ocellata* mitogenomes.

vs. H. kurti Ludwig, 1891

Due to the similar external morphology between *H. kurti* and a small specimen of *H. ocellata*, some scholars have regarded them as the same species (Liao and Clark 1995; Liao 1997). The sea cucumber *H. kurti* was first found by Sluiter (1889) and initially named "*Holothuria lamperti* Sluiter, 1889" (Ludwig 1891). Because of a naming duplication with "*Holothuria lamperti* Ludwig, 1886," Ludwig (1891) subsequently renamed the sea cucumber species as "*Holothuria kurti* Ludwig, 1891." In 1901, Sluiter described the ossicles of *H. kurti* for the first time and identified a table with cross-shaped chassis (synallactid-type table) as the characteristic of this species. Pearson (1913) compared the ossicles between *H. kurti* and *H. ocellata*, utilizing the differences in the types of tables as the basis for distinguishing the two species. Liao (1980, 1997) suggested that *H. kurti* might be the juvenile of *H. ocellata*, as he observed that the juveniles of *H. ocellata* had a similar synallactid-type table. In the meanwhile, he also found some large synallactid-type tables in the

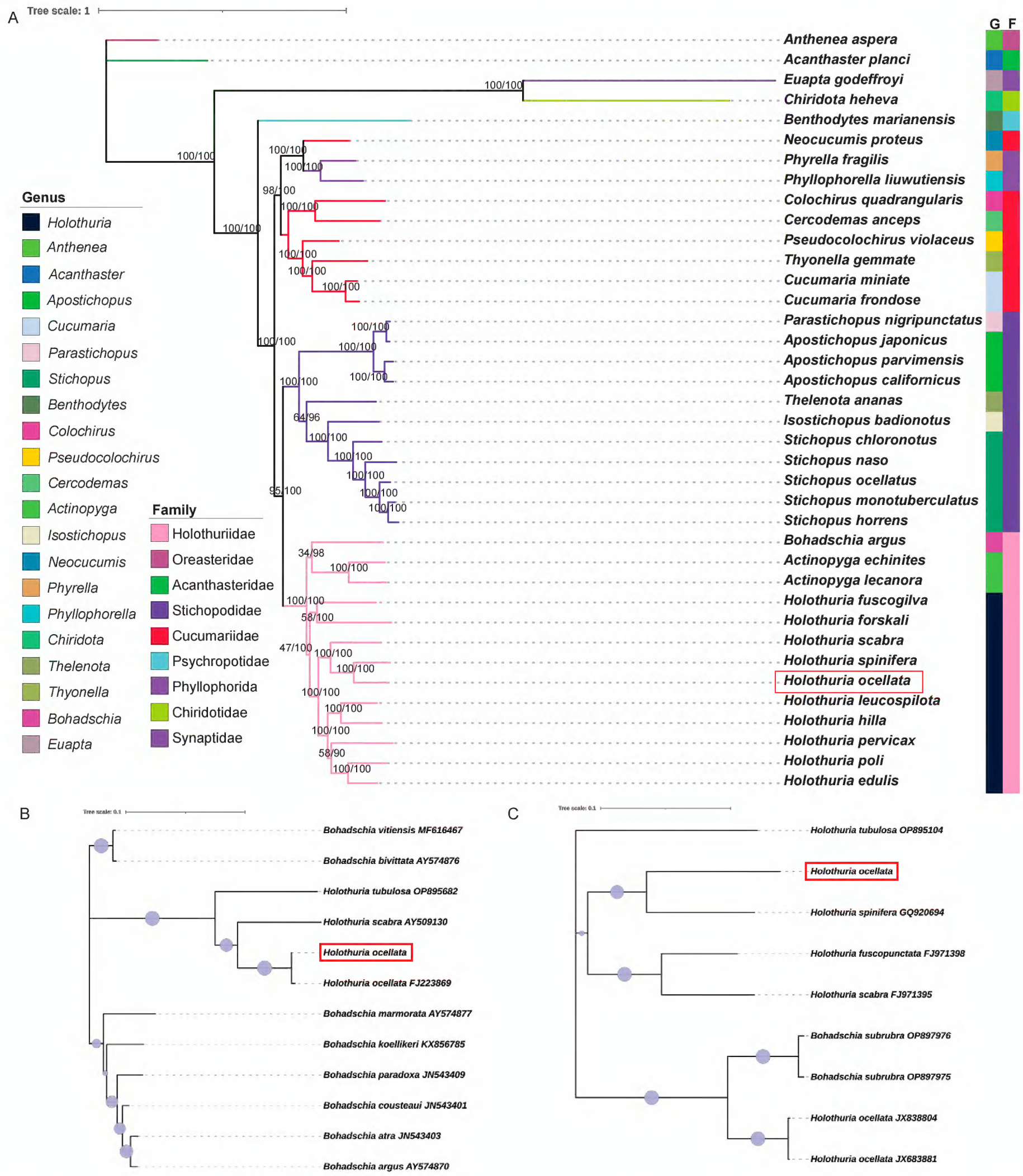

Figure 6. A. Phylogenetic tree of 36 Holothuroidea sequences constructed by Bayesian inference (BI) and maximum likelihood (ML) methods based on concatenated sequences of 13 PCGs. Asteroidea species were used as the outgroup; **B**. Phylogenetic tree of 12 *Holothuria* and *Bohadschia* sequences constructed by Neighbor-Joining (NJ) methods based on concatenated sequences of the *rrnL* gene; **C**. Phylogenetic tree of 9 *Holothuria* and *Bohadschia* sequences constructed by Neighbor-Joining (NJ) methods based on concatenated sequences of the *cox1* gene. The species in the red frame indicates the sequences generated in this study.

adults of *H. ocellata*. In previous studies, some sea cucumber species were also found to have their ossicles of adult individuals that were quite different from juvenile individuals (Gosliner et al. 1990; Massin et al. 2000; Soliman et al. 2019), which seemed to verify the credibility of Liao's conclusion. However, Samyn and Vandenspiegel (2016) disagreed with the conclusion; they collected some specimens of *H. kurti* and found that these samples were over 7 cm long and had mature gonads; they thought that *H. kurti* and *H. ocellata* were two different species. Besides, it is indeed true that scholars did not find tack-like tables in the papillae of *H. kurti*, which were found in the sample we collected in this study (Fig. 2O, P). For the above reasons,

we agree with the view of Samyn and Vandenspiegel (2016). It is reasonable to consider that *H. ocellata* and *H. kurti* are different species. We also hope that more molecular biological data on *H. kurti* will be available in the future to verify and support this conclusion.

***vs. H. hamata* Pearson, 1913**

The samples obtained in this study were similar in external form and ossicle types to the species found by Teo and Ng (2009), which they thought these samples were *H. ocellata*. However, some authors suggested that Teo and Ng's (2009) samples should be *H. hamata* (Aydin et al. 2019; Moazzam and Moazzam 2020). We do not agree with the point of Aydin et al. (2019) because: 1) *H. hamata*, as re-described by Aydin et al. (2019), has lateral papillae ± 20 in number on each side. However, *H. ocellata*, no matter which samples obtained from the current study or those of Teo and Ng (2009), exhibits approximately ± 40 lateral papillae on each side; 2) Tables of the body wall in *H. hamata* mainly have a spiny disc, whereas *H. ocellata* mainly has tables with a disc of smooth or wavy margin (Fig. 2E–H). Besides, Pearson (1913) first described the characteristics of both *H. hamata* and *H. ocellata* simultaneously. According to Pearson's records, *H. ocellata* had the Cuvierian tubules, while *H. hamata* did not. This discrepancy further certifies that these two sea cucumbers are different species.

Discussion on the classification status of Holothuria ocellata based on the mitochondrial genome

The complete mitochondrial genome of *H. ocellata* has a similar A-T content with the other holothuroid mitogenomes analyzed in the previous study (Utzeri et al. 2020; Sun et al. 2021; Li et al. 2022; Ma et al. 2022). The comparison of the gene order showed that *H. ocellata* has the same gene position on mtDNA as that of all other *Holothuria*, suggesting a common ancestral condition and a closer evolutionary relationship. The taxonomic controversy about *H. ocellata* centers on its subgenus: Jaeger (1833) initially thought it belonged to subgenus *Holothuria*, and some authors considered it as subgenus *Metriatyla* Rowe, 1969 (Rowe 1969; Cannon and Silver 1986). Moreover, some scholars have proposed that it should be classified into the subgenus *Theelothuria* based on the further study of ossicles (Liao 1997; Lane et al. 2000). The result of phylogenetic analysis indicated that *H. ocellata* preferentially forms a sister group with *H. spinifera* before clusters with *Holothuria scabra* Jaeger, 1833 (Fig. 6A). Besides, the result of phylogenetic analysis also indicated that *H. ocellata* preferentially forms a sister group with *H. spinifera* before clusters with *Holothuria tubulosa* Gmelin, 1791 (Fig. 6C). That means, compared to *H. scabra* and *H. tubulosa*, the member of subgenus *Metriatyla* and subgenus *Holothuria*, *H. ocellata* is more closely related to *H. spinifera*, which belongs to subgenus *Theelothuria*. In early studies, some scholars also found that *H. ocellata* is closely related to the species of subgenus *Theelothuria*: Théel (1886) considered *H. ocellata* to be doubtlessly nearly allied to *H. squamifera*; Pearson (1913) also found that *H. ocellata* and *H. spinifera* were exceedingly similar in morphology; and either *H. squamifera* or *H. spinifera*, both sea cucumber species, belong to subgenus *Theelothuria*. Because of that, it is reasonable to believe that *H. ocellata* should be classified within the subgenus *Theelothuria*.

The suggestion of formal Latin names about *H. ocellata*

Due to the lack of original descriptions and the difficulties in retrieving ancient references, identifying whether *B. ocellata* or *H. ocellata* refers to the same species discovered by Jaeger (1833) presents a challenge. Fortunately, Samyn and Vandenspiegel (2016) examined the holotype of *Bohadschia ocellata* Jaeger, 1833, and concluded that this is a valid *Bohadschia* species. In addition, the validity of the classification status of *B. ocellata* was certified by WoRMS. Because of that, we can assume that the species discovered by Jaeger was *Bohadschia ocellata*. Semper (1868) found a sea cucumber species and recorded it as *H. ocellata*, only citing Jaeger's descriptions without further explanation. Théel (1886), the third recorder of *H. ocellata*, not only gave a detailed description but also made hand-drawn pictures of appearance and ossicles. After comparing the description and the hand-drawn images of the species, we found them to be largely consistent with the samples obtained in this study. We speculated that Théel's (1886) record should be considered the actual first discovery of *H. ocellata*. According to the relevant provisions of the International Code of Zoological Nomenclature and considering the current classification of *H. ocellata*, we propose that it should be given a new formal Latin name. However, due to the absence of type specimens found by Théel (1886), it is not appropriate to give this species a new name directly by ourselves. Because of that, the full Latin name of this species should tentatively be "*Holothuria ocellata* Jaeger, 1833 *sensu* Théel 1886," which means "specimens belonging to the species that Théel 1886 called *Holothuria ocellata*" and distinguishes it from "*Bohadschia ocellata* Jaeger, 1833."

Conclusion

In this study, the complete mitochondrial genome of *H. ocellata* was characterized for the first time, and the morphology of this species, including its external form and ossicles, was further described. After careful consideration of the obtained data and comparison with previous references, we clarified the classification history and current status of *H. ocellata*. Finally, we suggest that the

full Latin name of this species should be "*Holothuria ocellata* Jaeger, 1833 *sensu* Théel 1886." The present study demonstrates the significant advantage of coupling ossicle data with molecular data to confirm species identification. Furthermore, the complete mitogenome sequence of *H. ocellata* Jaeger, 1833 *sensu* Théel 1886, will serve as a valuable genomic resource for further studies on holothuroid species. In addition, we recommend the continuation of broader taxonomy studies of holothuroids, employing a combination of "morphology + molecular analysis," to provide valuable information for taxonomy and evolutionary biology studies.

Acknowledgments

We sincerely thank the reviewer and editor for their critique and suggestions. At the same time, we are especially grateful to Dr. Elie Saliba and Dr. Zhengsen Yu for their detailed explanation and advice on the nomenclatural theory. This work was supported by the National Key Research and Development Program of China (2022YFD2401303), the Hainan Provincial Natural Science Foundation of China (321RC1023), and the National Natural Science Foundation of China (42166005; 42076097).

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Supplementary material 1

Additional information

Authors: Chenghao Jia, Zening Xu, Fengping Li, Liuna Chen, Aimin Wang, Fei Gao

Data type: tif

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